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Interactions between a luminescent conjugated polyelectrolyte and amyloid fibrils investigated with flow linear dichroism spectroscopy

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ABSTRACT

Luminescent conjugated polyelectrolytes (LCPs) have emerged as novel stains to detect and distinguish between various amyloidogenic species, including prefibrillar aggregates and mature fibril deposits, both *in vitro* and in histological tissue samples, offering advantages over traditional amyloid stains. We here use linear dichroism (LD) spectroscopy under shear alignment to characterize interactions between the LCP poly(3-thiophene acetic acid) (PTAA) and amyloid fibrils. The positive signature in the LD spectrum of amyloid-bound PTAA suggests that it binds in the grooves between adjacent protein side-chains in the amyloid fibril core, parallel to the fibril axis, similar to thioflavin-T and congo red. Moreover, using LD we record the absorption spectrum of amyloid-bound PTAA in isolation from free dye showing a red-shift by ca 30 nm compared to in solution. This has important implications for the use of PTAA as an amyloid probe *in situ* and *in vitro* and we demonstrate how to obtain optimal amyloid-specific fluorescence read-outs using PTAA. We use the shift in maximum absorption to estimate the fraction of bound PTAA at a given concentration. PTAA binding reaches saturation when added in 36 times excess and at this concentration the PTAA density is 4–5 monomer units per insulin monomer in the fibril. Finally, we demonstrate that changes in LD intensity can be related to alterations in persistence length of amyloid fibrils resulting from changes in solution conditions, showing that this technique is useful to assess macroscopic properties of these biopolymers.

1. Introduction

Proteins can, under some conditions, convert from their native forms into ordered fibrillar amyloid structures. Deposition of such amyloid fibrils in tissue is a pathological hallmark of several forms of disease, including severe neurodegenerative disorders such as Alzheimer's and Parkinson's disease [1]. Essentially any polypeptide chain can convert into amyloid fibrils and despite the diversity in amino acid sequence and native fold of the proteins from which they form, amyloid fibrils have surprisingly similar basic structures. They are commonly about 10 nm in diameter and can extend up to several microns in length, have unbranched, twisted, repetitive morphologies and share a basic structural fold denoted the cross-β motif [1,2]. Amyloid formation is not only associated with disease but has also been shown to have functional biological roles [3]. Furthermore, owing to their rigid and regular structure, amyloid fibrils are gaining attention as biomolecular scaffolds in nanotechnology, for example in self-assembly of conducting and luminescent nanowires [4,5].

Presence of amyloid fibrils *in vitro* and in tissue is often inferred from tinctorial properties of amyloid-specific dyes such as thioflavin-T (Th-T) or congo red [6,7]. In recent years, luminescent conjugated polyelectrolytes (LCPs) have emerged as novel amyloid probes with potential of recognizing not only mature amyloid fibrils and amyloid plaques [8] but importantly, and in contrast to conventional amyloid stains, also the prefibrillar assemblies that have been suggested to be the most important neurotoxic species in many protein misfolding diseases [9]. The optical properties of LCPs are intimately linked with the geometry of their conjugated backbone, and when an LCP binds to a biomacromolecule with distinct morphology, it displays characteristic spectral shifts and intensity changes in absorption and emission [10,11]. The physical basis of these shifts has been explained in terms of how restriction of the free rotation of the polymer backbone, due to planarization of the LCPs in their amyloid-bound state, extends the effective conjugation length, resulting in red-shifted absorption and emission [12]. Poly(3-thiophene acetic acid) (PTAA, Fig. 1C), the first LCP shown to interact with amyloid fibrils, has for example been used to monitor the kinetics of amyloid formation, [13] and subsequently also to fingerprint distinct amyloid subtypes in histological tissue samples in greater detail than with conventional amyloid stains [14,15]. The staining resolution and signal-to-noise ratio of

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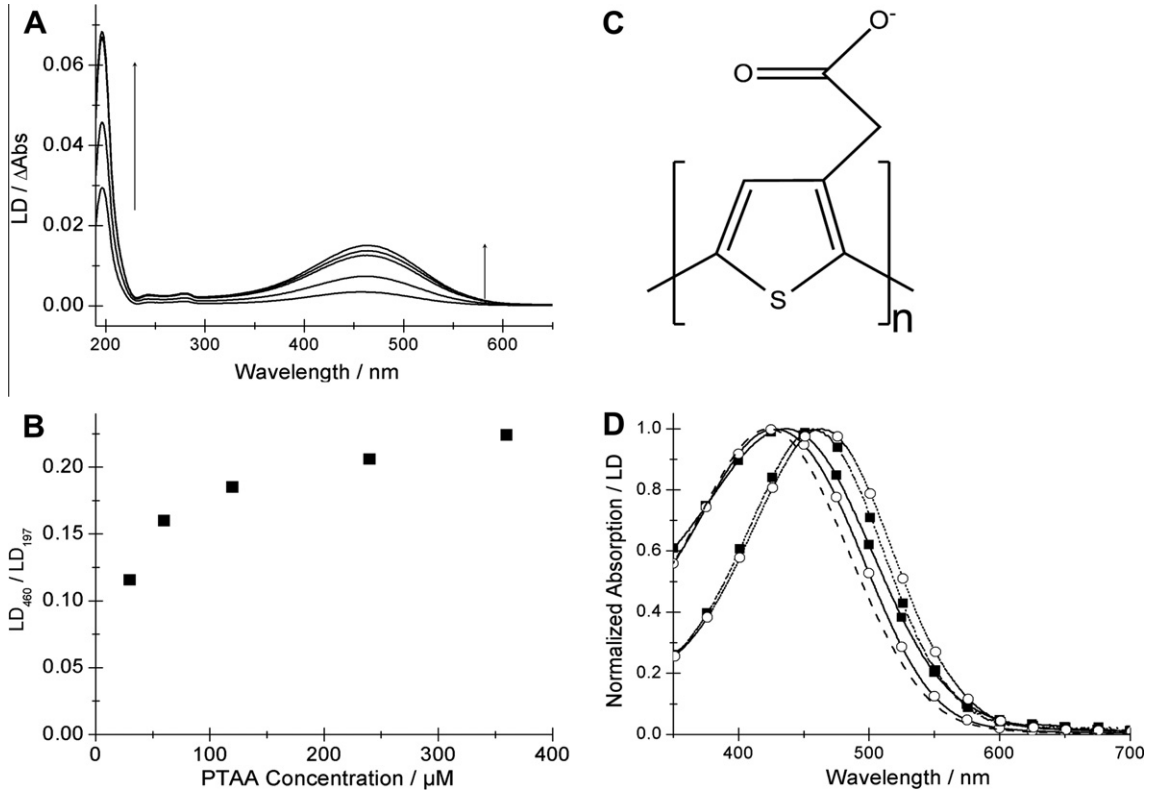

Fig. 1. (A) LD spectra of PTAA in presence of flow-aligned insulin amyloid fibrils (10 μM) in PB buffer (pH 7) at increasing PTAA concentrations (30, 60, 120, 240 and 360 μM). The arrows indicate direction of change. (B) The ratio of the LD signals at 460 and 197 nm. (C) Structure of the repeating unit in poly(3-thiophene acetic acid) (PTAA). (D). Normalized absorption (solid lines) and LD (dotted lines) spectra of PTAA bound to 10 μM amyloid fibrils at 30 (solid squares) and 360 (open circles) μM of PTAA. Dashed line is the normalized absorption spectrum of PTAA in absence of amyloid fibrils.

the PTAA emission has been reported to be superior to the birefringence from congo red in similar samples [15]. In addition, PTAA emits at longer wavelengths than for example Th-T and its emission is thus better separated from auto-fluorescence in surrounding tissue. A potential drawback of PTAA, particularly *in vitro*, is that it is fluorescent both in solution and in its amyloid-bound state. This complicates the isolated observation of amyloid-specific PTAA fluorescence and requires detailed knowledge of the optical characteristics of the amyloid-bound state.

Linear dichroism spectroscopy (LD) is an absorption technique that measures the differential absorption of linearly polarized light parallel and perpendicular to a macroscopic orientation axis. LD has been extensively used in combination with alignment in shear flow (flow-LD) to study DNA, [16] protein orientation in lipid membranes, [17] and recently also amyloid fibrils [18]. The sign and magnitude of individual absorption bands in an LD spectrum relate to the orientation of their corresponding transition moments. An LD spectrum will therefore provide information on the orientation of chromophores, both within the biopolymer itself and importantly in ligands that associate with the biopolymer. Molecules that are not bound to the aligned biopolymer remain isotropic and show no net LD. In contrast to other spectroscopic techniques, LD thus allows studies of the spectral characteristics of amyloid-bound ligands without interference from unbound ligands.

Here we use flow-LD of insulin amyloid fibrils to determine the binding geometry of PTAA. Further, from the isolated absorption spectrum of amyloid-bound PTAA, recorded using LD, we estimate the fraction of PTAA that is bound to the fibrils. The red-shifted absorption of bound PTAA is in agreement with previous descriptions on the effects of backbone planarization on the absorption spectrum of polythiophenes [19]. We also demonstrate how the absorption shift associated with amyloid binding effects the emission properties of PTAA and we discuss how an appropriate choice of excitation and emission settings can increase sensitivity and amyloid-specific read-out in fluorescence-based applications. As the thiophene backbone motif is common to most LCPs that have been used as amyloid stains, [8] our approach to dissect the absorption and emission properties of the amyloid-bound state may prove useful for optimization of other LCPs, and also aid in design of new amyloid-specific LCPs with improved characteristics.

2. Material and methods

2.1. Sample preparation

PTAA was synthesized as reported previously [20]. Stock solutions (1 mg ml^{-1}, 7.194 mM on a monomer basis) were prepared from lyophilized powder in mQ water with addition of sodium hydroxide until PTAA was fully dissolved. Phosphate buffer (PB) pH 7.4 (50 mM Na_2HPO_4) and glycine–hydrochloride buffer (Gl–HCl) pH 2.2 (50 mM) were prepared in mQ water and filtered (0.4 μm). Amyloid fibrils from bovine insulin (Sigma–Aldrich) were formed by heating an insulin solution (10 mg ml^{-1}) in Gl–HCl buffer (pH 2.2) at 60 °C over night. Large gel particles in the sample were removed by centrifugation in a tabletop centrifuge for a few seconds, resulting in a clear supernatant containing amyloid fibrils. The fibril concentration was determined using absorption spectroscopy. The extinction coefficient for insulin at 280 nm is ε_{280} = 5480 M^{-1} cm^{-1}. Amyloid fibrils were, if not stated otherwise, mixed with PTAA at pH 2.2 and thereafter diluted in PB buffer to reach the final sample concentration at pH 7. All amyloid and LCP concentrations throughout the paper are given on a monomer basis.

2.2. Flow linear dichroism

Linear dichroism (LD) is defined as the difference in absorbance of linearly polarized light parallel and perpendicular to an orientation axis. Sample orientation is achieved by shear flow in an outer rotating cylinder quartz Couette cell. LD spectra were measured on a Chirascan CD spectropolarimeter equipped with an LD.3 linear dichroism detector (Applied Photophysics), run in linear dichroism mode. A non-oriented sample was used as baseline. For a general introduction to polarized light spectroscopy we refer to a recent book [21].

2.3. Spectroscopic measurements

Emission and excitation spectra were recorded on a wavelength corrected SPEX Fluorolog t-3 spectrophotometer (Jobin Yvon Horiba). Absorption measurements were carried out on a Varian Cary 5000 (Agilent Technologies).

3. Results and discussion

Fig. 1A. shows LD spectra of PTAA bound to flow aligned bovine insulin amyloid fibrils recorded at increasing concentrations of PTAA. The samples were prepared by adding PTAA to a solution of insulin amyloid fibrils at pH 2.2. The sample was diluted to pH 7 where PTAA has optimal affinity for the amyloid fibrils [22]. The LD band observed above 400 nm originates from absorption along the PTAA backbone [23]. The positive sign of this band shows that PTAA binds preferentially along the amyloid fibril long axis, parallel to the orientation axis in the sample. The LD intensity from PTAA increases steadily with increasing PTAA-to-amyloid ratio up to concentrations around 100 μM, where it levels off in accord with saturable binding.

Concomitant with the increased LD from PTAA we observe an increase in LD signal in the absorption band centered at 197 nm. This band originates from absorption of the peptide bond amide chromophores whose transition moments are oriented parallel to the fibril axis [18]. The magnitude of this peak depends on the amyloid concentration and β-sheet content in the fibril, but there is also a linear dependency on the degree of macroscopic orientation in the sample and hence on how well the fibrils align in the shear flow. The LD spectrum of amyloid fibrils in presence of PTAA at pH 7 has identical shape to that of amyloid fibrils alone at pH 2.2 below 300 nm where the peptide backbone and aromatic side-chains absorb (Fig. S1, Supporting Information). This suggests that fibril secondary structure (peak at 197 nm) and tyrosine side-chain orientations (peaks at 230 and 276 nm) are not affected by the presence of PTAA. The weaker intensity in the UV-region of the LD spectra recorded at pH 7 is thus not due to changes in fibril structure. Instead we suggest that insulin amyloid fibrils that have been transferred from pH 2.2 to pH 7 become less rigid and possibly even condensed as they pass through their isoelectric point (pH 5.3 [24]), resulting in poorer alignment in the shear flow. TEM images of amyloid fibrils in absence of PTAA at pH 2 and in presence of PTAA at pH 7 reveal no significant changes in overall morphology and length distributions, but some large aggregates were detected in the latter case (Fig. S2, Supporting Information). In conclusion, we believe that there are two factors that contribute to the increase in LD signal of PTAA in the visible region upon titration with PTAA (Fig. 1A); increasing amounts of PTAA bind to the amyloid fibrils and concomitantly the fibrils become more rigid and therefore better aligned in the shear flow. In Fig. 1B we plot the ratio of the PTAA LD (at 460 nm) and the amyloid backbone LD (at 197 nm) as a function of the total PTAA concentration to obtain a binding curve corrected for the concomitant increase in amyloid alignment.

Fig. 1D. shows normalized isotropic absorption spectra corresponding to the LD spectra in Fig. 1A. at the highest and lowest PTAA concentrations. The isotropic absorption is significantly blue-shifted compared to the LD but it clearly represents a mixture of bound and unbound PTAA as it is red-shifted compared to the spectrum of PTAA in solution. The normalized LD spectra, representing amyloid-bound PTAA, are red-shifted ca 30 nm compared to the corresponding isotropic absorption spectra. This shift is significantly larger than what is discernable in the absorption measurements and larger than what has been previously reported [13]. The red-edge broadening of the isotropic absorption spectrum is most significant at low PTAA-to-amyloid ratios, where a larger fraction of the total added PTAA is bound. The marked difference in maximum wavelength in the LD spectrum (bound PTAA) and the isotropic absorption spectrum (both bound and free PTAA), even at the lowest PTAA concentration, shows that a significant fraction is not bound to the insulin amyloid fibrils and that the binding affinity is moderate to low. This is confirmed by the titration in Fig. 1B where at least a 30 times excess of PTAA (on a monomer basis) is needed to saturate binding. Comparison of the two normalized LD-spectra in Fig. 1D reveals that the absorption of bound PTAA is slightly red-shifted when binding approaches saturation (the difference is approximately 7 nm between 30 and 360 μM). This may indicate interactions between adjacent PTAAs in the amyloid-bound state as the fibrils become saturated that could result in further planarization of the PTAA backbone due to stacking. Alternatively the interactions give rise to exciton couplings of parallel or head-to-tail stacking type [25]. Both these phenomena are consistent with a red-shifted absorption and we cannot distinguish between the two at this point. Nevertheless, the bound PTAA polymers appear to come in close contact at concentrations above 100 μM which agrees with the saturation observed in Fig. 1B.

Furthermore, we used the data in Fig. 1D. to estimate the fraction of bound PTAA at a given concentration. Under the assumption that the isotropic absorption maximum red-shifts roughly linearly with the increase in fraction bound we used the absorption maxima of the LD spectrum and the isotropic absorption spectrum of free PTAA as references to determine the fraction of bound PTAA from the absorption maximum. Our calculations suggest that of the total PTAA added 39% at 30 μM PTAA and 13% at 360 μM PTAA is bound. We successfully reconstructed the isotropic absorption spectra of the samples at these two concentrations as a weighted sum of the reference spectra giving further validity to our approach (Fig. S3, Supporting Information). At the higher concentration, when the fibrils are saturated with PTAA (Fig. 1B), the bound fraction corresponds to 4–5 PTAA monomer units per insulin monomer. As comparison, the length of one repeating PTAA monomer is 3.8 Å and the distance between β-strands in the amyloid fibril is 4.6–4.8 Å [26].

Th-T [6] and congo red [7], have both been suggested to bind in the grooves that form in-between adjacent amino acid side-chains, extending from the same side of the β-sheet in the amyloid fibril core. These grooves are 6.5–7 Å wide [27] and run along the axis of the amyloid fibril. The spatial dimensions of these grooves put sterical constraints on the possible conformations that dyes with molecular rotor properties can adopt when bound, and a planar conformation is favored. Since our data show that PTAA binds parallel to the amyloid fibril long axis and displays a red-shifted absorption in its bound state, we suggest that the binding occurs in these grooves, similar to Th-T and congo red. Moreover, as binding of one PTAA polymer will involve it covering several monomer units in the amyloid fibril this gives a reasonable explanation to its amyloid specificity, as similar arrangement of β-sheets are not

found in globular proteins. Our estimate of the number of PTAA monomer units per insulin monomer in the fibril (see above) suggests that PTAA must bind to several grooves and saturation is possibly primarily related to how many PTAA molecules that can be accommodated.

Insulin amyloid fibrils transferred from pH 2.2 to pH 7 in absence of PTAA lose their intrinsic ability to align in shear flow and hence do not show any LD signal. We suggest that this is due to an "electrostatic collapse" of the amyloid fibrils when the solution passes through the isoelectric point of insulin. This observation demonstrates how LD can be used to explore mechanical properties and condensation behavior of amyloid fibrils, material characteristics that are important both for understanding of their stabilities *in vivo* and also their use as scaffolds in nanotechnology applications. In presence of PTAA this collapse is prevented and LD spectra can be recorded at pH 7. We believe that this is because PTAA binding increases the isoelectric point of the amyloid fibrils. To further explore the relations between degree of amyloid fibril alignment, solution pH and potential stabilizing or stiffening properties of PTAA we investigated if presence of PTAA would lead to that insulin amyloid fibrils regain alignment in shear flow at pH 7. Indeed, the LD signals from both PTAA and the amyloid fibrils increase with time after addition of 120 μM PTAA to a 10 μM solution of insulin amyloid fibrils at pH 7 (Fig. 2A). The signal recovery is however slow and even after 20 h incubation the amyloid LD-signal is only about 40% of that in a sample where the same concentration of PTAA was added at pH 2.2 prior to raising the pH (Fig. 2B).

As discussed above, PTAA is fluorescent both in solution and when bound to amyloid fibrils which complicates data analysis, particularly for *in vitro* experiments where unbound dye cannot be easily removed. Our observation that PTAA's affinity for amyloid fibrils is moderate shows that it is important to take this into account to obtain amyloid-specific read outs. The new knowledge, gained from LD spectroscopy, regarding the absorption properties of the amyloid-bound fraction of PTAA is useful to better describe the emission properties in samples containing amyloid fibrils and PTAA. Fig. 3A shows emission spectra of PTAA in absence and presence of amyloid fibrils recorded at increasing excitation wavelengths (375–550 nm). As the excitation wavelength is increased the intensity difference between each set of spectra becomes more pronounced (Fig. 3B). This is fully consistent with the data in Fig. 1 and shows that it is possible to almost exclusively excite the amyloid-bound fraction at wavelengths above 500 nm.

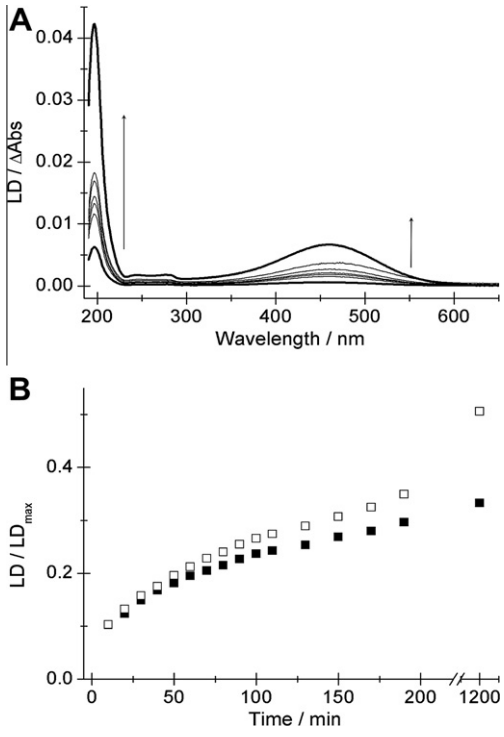

Fig. 2. (A) LD spectra recorded at 0 (thick line, lowest intensity), 30, 60, 90, 190 min, and 20 h (thin lines, concentration increase in direction of arrow) after addition of 120 μM PTAA to 10 μM amyloid fibrils at pH 7. Thick line, highest intensity, is a sample where 120 μM PTAA was added at pH 2 and the sample thereafter diluted with PB buffer to pH 7. (B) The LD intensity as function of time at 460 nm (open squares) and 197 nm (solid squares).

Normalized excitation spectra of PTAA with and without insulin amyloid fibrils, recorded at increasing emission wavelengths, are shown in Fig. 3C. There is a significant red-shift and broadening of the excitation spectrum with increasing emission wavelength in presence of amyloid fibrils, consistent with excitation spectra reported in the literature [14]. The growing shoulder on the red side of the spectra coincides perfectly with the absorption profile of amyloid-bound PTAA obtained in the LD-spectra, confirming that it originates exclusively from the amyloid-bound PTAA-fraction. This shoulder is much more distinct in the excitation spectrum compared to the isotropic absorption spectrum (Fig. 1D) which shows that amyloid-bound PTAA has a significantly higher emission quantum yield.

The demonstration of how the emission properties of PTAA depend on the configuration ensemble of excited PTAA molecules shows that if we explore in detail the absorption and emission properties of PTAA bound to amyloid fibrils and in the future also to other types of amyloid aggregates, including protofibrils and oligomers, we will be able to improve the sensitivity of existing LCPs significantly simply by a careful choice of excitation and emission settings. For PTAA in particular we show that using excitation wavelengths above 475 nm, such as the standard 488 nm Argon laser line available in virtually all confocal microscopes, should lead to a sufficient distinction between bound and free fractions. Excitation at even longer wavelengths (515 or 543 nm for example) would significantly improve this selectivity, but may decrease image contrast due to weaker over-all emission intensities.

In conclusion, we have used flow-LD spectroscopy to examine the absorption spectrum of amyloid-bound PTAA, without spectral overlap from unbound PTAA in solution, obtaining novel information on the exact spectral properties of this state. Our data shows that the bound PTAA fraction is red-shifted by approximately 30 nm compared to PTAA in solution, consistent with that PTAA is planarized in its amyloid-bound state. The positive sign of the LD shows that the PTAA backbone binds parallel to the amyloid fibril long axis and we suggest that PTAA inserts into the grooves formed between adjacent amino acid side-chains that run along the amyloid fibril axis. Similar binding modes have been proposed for Th-T and congo red, suggesting that their selectivity for amyloid fibrils have a common basis. We use the LD-spectrum and the isotropic absorption spectrum of free dye as references to approximate the fraction of PTAA that is bound to the amyloid fibrils and show that 4–5 PTAA monomer units are bound per insulin monomer at saturation. We show that it is possible to obtain up to an order of magnitude difference in emission intensity between amyloid-bound and free PTAA by exciting the sample at wavelengths above 500 nm. This observation has implications for fluorescence microscopy where the contrast between bound and free PTAA can be significantly increased by choosing an appropriate excitation wavelength. We also show that flow-LD can be used to sense the extent of orientation in an amyloid fibril sample and that pH and/or presence of PTAA significantly affects the macroscopic properties of insulin amyloid fibrils. In particular, PTAA can solubilize aggregated fibrillar assemblies, which may have important

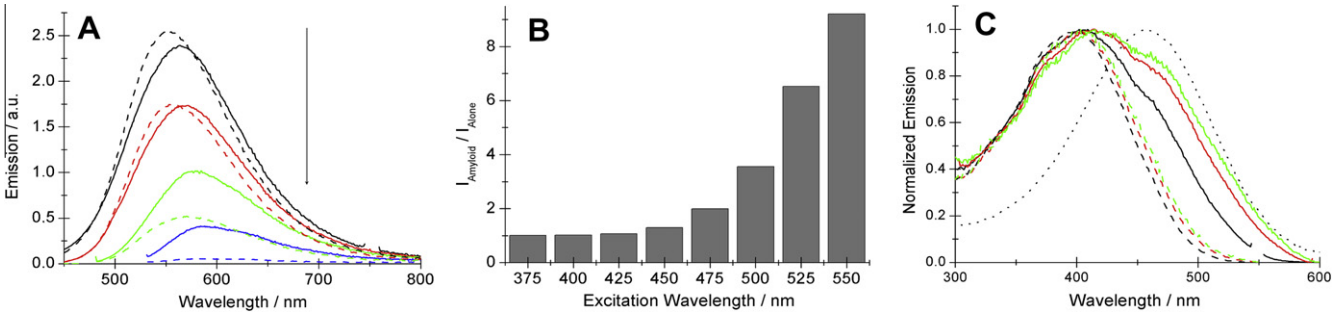

Fig. 3. (A) Emission spectra of PTAA in absence (dashed lines) and presence (solid lines) of amyloid fibrils. Spectra recorded with excitation at 375 nm (black), 425 nm (red), 475 nm (green) and 525 nm (blue). The associated intensity change is indicated by the direction of the arrow. (B) Ratio between the integrated emission intensity with ($I_{Amyloid}$) and without (I_{Alone}) amyloid fibrils as a function of excitation wavelength. (C) Excitation spectra of PTAA without (dashed lines) and with (solid lines) amyloid fibrils. Emission collected at 550 nm (black), 600 nm (red) and 650 nm (green). The dotted black line is the normalized LD-spectrum of amyloid-bound PTAA. Spectra recorded in PB buffer at pH 7 with 5 μM amyloid and 15 μM PTAA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

consequences for *in situ* staining as our data suggests that PTAA could modulate the properties of amyloid plaques, which calls for caution as PTAA may distort the morphological information obtained. This study also demonstrates the power of flow aligned LD as a technique to monitor amyloid fibrils and their interactions with LCPs, that also holds promise for studies of other amyloid-specific ligands, including small-molecule drugs and potentially also chaperone proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.132.

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